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Effect of carbohydrate-protein supplementation postexercise on rat muscle glycogen synthesis and phosphorylation of proteins controlling glucose storage

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ABSTRACT

To examine whether addition of protein to a carbohydrate supplement enhances muscle glycogen synthesis, we compared the muscle glycogen concentrations of rats that had been depleted of their muscle glycogen stores with a 3-hour swim and immediately supplemented with a placebo (Con), carbohydrate (CHO), or carbohydrate plus protein supplement (C+P). Rats were given either 0.9 g carbohydrate per kilogram body mass for the CHO group or 0.9 g carbohydrate + 0.3 g protein per kilogram body mass for the C+P groups. Muscle samples of the red and white quadriceps were excised immediately, 30 minutes, or 90 minutes postexercise. Glycogen concentration of the C+P group was greater than that of the CHO group at 90 minutes postexercise in both red (C+P, 28.3 ± 2.6 μmol/g vs CHO, 22.4 ± 2.0 μmol/g; $P < .05$) and white (C+P, 24.9 ± 2.4 μmol/g vs CHO, 17.64 ± 1.5 μmol/g; $P < .01$) quadriceps. Protein kinase B phosphorylation was greater in the C+P-30 group (the number following treatment group abbreviation refers to time [in minutes] of euthanasia following exercise) than the sedentary control and exercised control groups in red quadriceps at 30 minutes and in white quadriceps at 90 minutes postexercise. This difference was not observed in the CHO group. Phosphorylation of glycogen synthase was significantly reduced 30 minutes postexercise and returned to baseline levels by 90 minutes postexercise in both CHO- and C+P-supplemented groups, with no difference between supplements. These results demonstrated that the addition of protein to a carbohydrate supplement will enhance the rate of muscle glycogen restoration postexercise and may involve facilitation of the glucose transport process.

1. Introduction

Muscle glycogen is a major fuel source during moderate- to high-intensity prolonged exercise. As the intensity of exercise increases, the reliance on this intramuscular energy source increases. When muscle glycogen is depleted, humans and animals are not able to perform moderate- to high-intensity exercise continuously. Therefore, the depletion of muscle glycogen is highly related to fatigue. To improve endurance performance, researchers have mainly focused on nutritional

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supplementation before and during exercise. Postexercise nutrition has received less attention, even though recovery is a critical aspect of training and the degree of muscle glycogen repletion after exercise may affect the quality of the next bout of exercise. Ivy [1] found that the rate of muscle glycogen synthesis could be maximized by ingesting a carbohydrate supplementation of 1.2 to 1.5 g/kg body mass immediately postexercise and at 2-hour intervals. However, more recent research suggests that glycogen replenishment can be further enhanced by the addition of protein to a carbohydrate supplement [2-5].

The mechanism by which the addition of protein to a carbohydrate supplement enhances muscle glycogen storage is not clearly understood. One possible mechanism is that carbohydrate plus protein supplementation has a greater effect on insulin secretion than carbohydrate alone [4,6,7]. In a glycogen-depleted muscle cell, insulin is not required during the initial phase (30-60 minutes) of muscle glycogen synthesis; however, insulin is necessary for glycogen synthesis after this short insulin-independent phase [8].

Because glycogen synthase is the rate-limiting enzyme for glycogen synthesis and this protein is a downstream target of the insulin-signaling pathway, a greater insulin secretion due to carbohydrate and protein supplementation may have a stimulatory effect on this enzyme. Whereas early studies reported that carbohydrate and protein supplementation resulted in a greater insulin secretion compared with carbohydrate alone, Ivy et al [3] did not observe any difference in insulin response between those 2 types of supplements, but still found an increased rate of glycogen synthesis after carbohydrate plus protein supplementation. Therefore, a greater insulin concentration per se may not be the main reason for the difference in the rate of glycogen synthesis. An alternative hypothesis is that the addition of protein (amino acids) to a carbohydrate supplement may have an additive effect on enzymes controlling glycogen storage.

Although several studies have observed differences in glycogen synthesis between carbohydrate-plus-protein supplementation and carbohydrate-alone supplementation after recovery from a glycogen-depleting exercise [2-4], inconsistent results have also been reported [9-13]. Therefore, the first purpose of this study was to compare muscle glycogen concentrations of rats 30 and 90 minutes postexercise when supplemented immediately postexercise with either a carbohydrate or carbohydrate-plus-protein supplement. The second purpose of the study was to investigate the differences between carbohydrate and carbohydrate-plus-protein supplementation on the phosphorylation status of proteins involved in glycogen synthesis.

2. Materials and methods

Male Sprague-Dawley rats weighing 200 to 250 g (N = 49) were obtained from the Animal Resource Center at the University of Texas at Austin. They were housed in a room maintained on a 7:00 AM to 7:00 PM light cycle and at a temperature of 21°C. The rats were allowed free access to water and rat chow (Purina chow; Ralston Purina, St Louis, MO), except when indicated. All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin.

2.1. Experimental procedures

On the day of each experiment, food was withdrawn 9 hours before the start of the swimming exercise. The rats were randomly assigned to one of the following 4 experimental groups: sedentary control (SED-Con), exercise alone (EX), exercise-carbohydrate (CHO), and exercise-carbohydrate plus protein (C+P). The CHO and C+P groups were further subdivided into 2 different groups based on time of euthanasia: 30 and 90 minutes postexercise. The EX group was subdivided into 3 groups: control (EX-Con), 30 minutes postexercise (EX-30), and 90 minutes postexercise (EX-90). There were a total of 8 experimental groups. Each group had 6 rats except for the SED-Con group, which had 7 rats. All rats were familiarized with the swimming environment for 10 min/d for 2 days. The exercise protocol consisted of a 3-hour swimming exercise with a weight equal to 3% of the rat's body weight attached approximately one third from the distal end of the tail. The swimming exercise was performed in a barrel, which was filled with water to a depth of 50 cm. The inside diameter of the barrel was 50 cm. Two rats swam in a barrel at the same time. The water temperature was maintained at 33°C to 34°C. If any rats fatigued during the exercise, a 5- to 10-minute rest period was allowed. If any rats could not keep swimming for 10 minutes, weight on the tail was reduced by 0.5% of body weight; and a 5-minute rest was given. Immediately after exercise, the rats were dried with a towel and intubated with a liquid supplement. The CHO groups received 0.9 g carbohydrate per kilogram; and the C+P groups received 0.9 g carbohydrate per kilogram + 0.3 g protein per kilogram, or approximately 25% more calories than the CHO groups in the form of protein. The concentrations of liquid supplements were 23.7% wt/vol and 23.7% wt/vol carbohydrate + 7.9% wt/vol protein for CHO and C+P, respectively. The carbohydrate consisted of dextrose, and the protein consisted of a whey isolate. The EX groups did not receive any supplement after the exercise protocol. Rats in the SED-Con and EX-Con groups were killed immediately after a 3-hour rest or 3-hour swim, respectively. The treatment groups (CHO, C+P, and EX) were killed either at 30 or 90 minutes postexercise. Approximately 8 minutes before the surgery, all rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65.0 mg/kg body mass). Approximately 1 mL of blood was withdrawn from the tail vein of the rats immediately before muscle excision. Superficial (white) and deep (red) portions of the quadriceps, which consist predominantly of fast-twitch white fibers and fast-twitch red fibers, respectively [14], were excised from both legs; and the muscles were clamped frozen with tongs cooled in liquid nitrogen and stored at –80°C for later analysis.

2.2. Blood assays

At the onset of each blood draw, one drop of blood was used to measure glucose using the One Touch Basic glucose analyzer (Johnson & Johnson, Milpitas, CA). Once this was performed, approximately 1 mL of blood was withdrawn in a 1.5-mL

microcentrifuge tube containing 20 μL of EDTA (24 mg/mL, pH 7.4) and centrifuged at 14 000*g* for 10 minutes. The plasma was stored at –80°C subsequent to plasma insulin determination by ^{125}I radioimmunoassay (MP Biomedicals, Orangeburg, NY).

2.3. *Muscle glycogen*

Muscle glycogen concentration was determined by a modification of the method by Passonneau and Lauderdale [15]. About 50 mg of muscle was dissolved in 1 N KOH at 70°C for 30 minutes. The dissolved homogenate, 0.1 mL, was added to 0.25 mL 0.3 mol/L sodium acetate buffer (pH 4.8) and 0.01 mL of 50% glacial acetic acid and incubated overnight at room temperature after adding an additional 0.25 mL of 0.3 mol/L sodium acetate buffer containing 10 mg/mL amyloglucosidase. Following incubation, the reaction mixture was neutralized with 2 N NaOH. Concentration of glucose was analyzed spectrophotometrically using a glucose color reagent (Raichem, San Diego, CA).

2.4. *Western blotting*

Approximately 60 mg of the frozen muscle samples were homogenized in ice-cold buffer (1:9 wt/vol; pH 7.4) containing 20 mmol/L 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 2 mmol/L ethylene glycol tetraacetic acid, 50 mmol/L NaF, 100 mmol/L KCl, 0.2 mmol/L EDTA, 50 mmol/L glycerol phosphate, 1 mmol/L DL-dithiothreitol, 0.1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L benzamidine, and 0.5 mmol/L Na vanadate (1 mL/100 mg muscle) with a glass tissue grinder and pestle (Corning Life Sciences, Acton, MA). The homogenate was centrifuged at 14 000*g* for 10 minutes at 4°C. Thereafter, aliquots of the supernatant were stored at –80°C for later analysis. The homogenate protein concentration was measured using the method of Lowry et al [16].

Aliquots of homogenized muscle samples and standards were slowly thawed over ice and diluted 1:1 with Laemmli sample buffer (pH 6.8) containing 125 mmol/L Tris base, 20% glycerol, 2% sodium dodecyl sulfate, and 0.008% bromophenol blue and then boiled for 5 minutes [17]. They were then analyzed via Western blotting for protein kinase B (PKB), glycogen synthase kinase (GSK)-3α/β, glycogen synthase (GS), and α-tubulin as previously described [18]. The membranes were visualized by enhanced chemiluminescence (Perkin Elmer, Boston, MA), and the density of each band was quantified using Scion Image version 4.0.3 (Scion, Frederick, MD). All blots were compared with a standard made from insulin-stimulated rat skeletal muscle, and the percentage of the standard was calculated. Evaluation of α-tubulin, which was used as a housekeeping protein, indicated no differences in protein loading across all treatments.

2.5. *Statistical analysis*

All values are expressed as means ± standard error of the mean (SE). A 1-way analysis of variance was used to test for significant differences among the treatments. For each dependent variable, post hoc tests were performed using Fisher least significant difference to identify significant differences between group means. A level of $P \leq .05$ was set a priori for statistical significance for all tests. Statistical analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL).

3. Results

3.1. *Blood glucose*

Three hours of swimming exercise resulted in a 60% reduction in blood glucose concentration (Fig. 1A). Recovery of blood glucose was not observed during the first 30 minutes postexercise if nutritional supplementation was not provided. However, a significantly greater glucose concentration was observed in the EX-90 group compared with the EX-Con and EX-30 groups. The blood glucose concentration of the EX-90 group was, however, still 21% lower than that of the SED-Con group ($P < .05$). Although the nutritionally supplemented groups did not recover blood glucose concentration to that of the SED-Con group by 30 minutes postexercise (CHO-30 and C+P-30), these groups had significantly greater blood glucose concentrations compared with the EX-Con and EX-30 groups. By 90 minutes postexercise, blood glucose concentrations of the CHO-90 and C+P-90 groups were significantly greater than those of the EX-Con, EX-30, and C+P-30 groups and were similar to that of the SED-Con group.

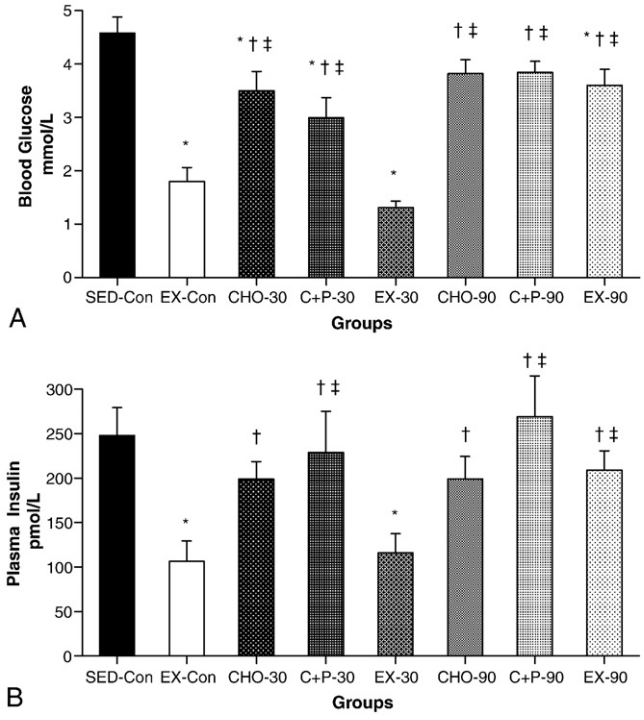

Fig. 1 – Blood glucose (A) and plasma insulin (B) concentrations for SED-Con and exercised rats. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, †from EX-Con, and ‡from EX-30.

3.2. Plasma insulin

The swimming exercise resulted in a significant ($P < .05$) reduction in plasma insulin as indicated by the lower concentration in the EX-Con group compared with the SED-Con group (Fig. 1B). There were no differences in plasma insulin concentration between the EX-Con and EX-30 groups, and the EX-30 group had significantly lower plasma insulin concentration compared with the SED-Con group ($P \leq .01$). However, a significant elevation of plasma insulin was demonstrated in the EX-90 group compared with the EX-Con and EX-30 groups. Moreover, there were no differences in plasma insulin concentrations between the SED-Con group and the EX-90 group. The CHO-30 and C+P-30 groups had significantly greater insulin concentrations compared with the EX-Con group. Although the blood glucose concentrations at 30 minutes postexercise were significantly greater in the groups that received nutrient supplements compared with the EX-30 group, only the C+P-30 group had a significantly greater plasma insulin concentration compared with the EX-30 group ($P \leq .05$). Plasma insulin concentrations of the CHO-90 and C+P-90 groups were significantly greater than that of the EX-Con group. Furthermore, the C+P-90 group had significantly greater plasma insulin concentration compared with the EX-30 group.

3.3. Glycogen concentration

Following swimming exercise, muscle glycogen concentrations in both red (Fig. 2A) and white quadriceps (Fig. 2B) of the EX-Con group were reduced 48.0% and 47.1%, respectively, compared with the SED-Con group ($P < .01$). Without nutritional supplementation, glycogen concentrations were not different among the EX-Con, EX-30, and EX-90 groups in either red or white quadriceps. No significant differences in glycogen concentration were observed among the 30-minute postexercise groups in red quadriceps. Glycogen concentrations of 30-minute postexercise groups were similar to that of the EX-Con group and significantly lower than that of the SED-Con group in red quadriceps. However, the C+P-90 group had a significantly greater glycogen concentration compared with all other treatments except for the SED-Con group in red quadriceps. The CHO-90 group demonstrated a greater glycogen concentration compared with all groups except for the SED-Con and C+P-90 groups, with its glycogen concentration 20.8% lower than that of the C+P-90 group and 31.0% lower than that of the SED-Con group in red quadriceps.

In the white quadriceps, there were no differences in glycogen concentrations among the 30-minute postexercise groups; and glycogen concentrations of these groups were significantly lower than that of the SED-Con group (Fig. 2B). Glycogen concentration of the CHO-90 group was significantly lower than those of the SED-Con ($P \leq .01$) and C+P-90 ($P \leq .05$) groups but significantly greater than that of the C+P-30 group. Whereas the C+P-90 group had greater glycogen storage compared with all other exercise groups including the CHO-90 group, the glycogen concentration of this group was still 23.2% lower than that of the SED-Con group in white quadriceps ($P \leq .05$).

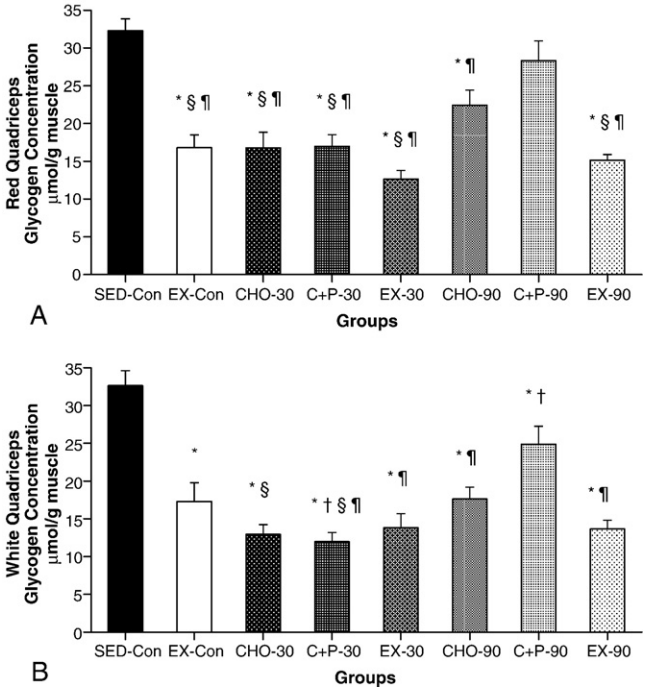

Fig. 2 – Muscle glycogen concentrations in red (A) and white (B) quadriceps for SED-Con and exercised rats. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, †from EX-Con, §from CHO-90, and ¶from C+P-90.

3.4. Protein kinase B

No effect on the PKB Ser473 phosphorylation was observed immediately after 3 hours of swimming exercise in either red (Fig. 3A) or white quadriceps (Fig. 3B) compared with the SED-Con group. Without nutritional supplementation, an exhaustive exercise had no effect on the PKB phosphorylation at 30 minutes postexercise in either red or white quadriceps. However, a significant elevation of the PKB phosphorylation in the EX-90 group compared with the SED-Con and EX-Con groups was observed in both red and white quadriceps. Furthermore, the phosphorylation of PKB in the EX-90 group was significantly greater than that of the C+P-90 group and those of the C+P-30 and EX-30 groups in red quadriceps and white quadriceps, respectively. Although no significant differences in the PKB phosphorylation were observed among the 30-minute postexercise groups, significantly greater PKB phosphorylation was found in the C+P-30 group compared with the SED-Con ($P \leq .01$) and EX-Con ($P \leq .01$) groups in red quadriceps. However, this increased PKB phosphorylation was not observed in the red quadriceps of the C+P-90 group.

In the white quadriceps, PKB phosphorylations for the CHO-30, C+P-30, and EX-30 groups were similar to and not different than SED-Con or Ex-Con treatments. Likewise, there were no differences in the PKB phosphorylation among the

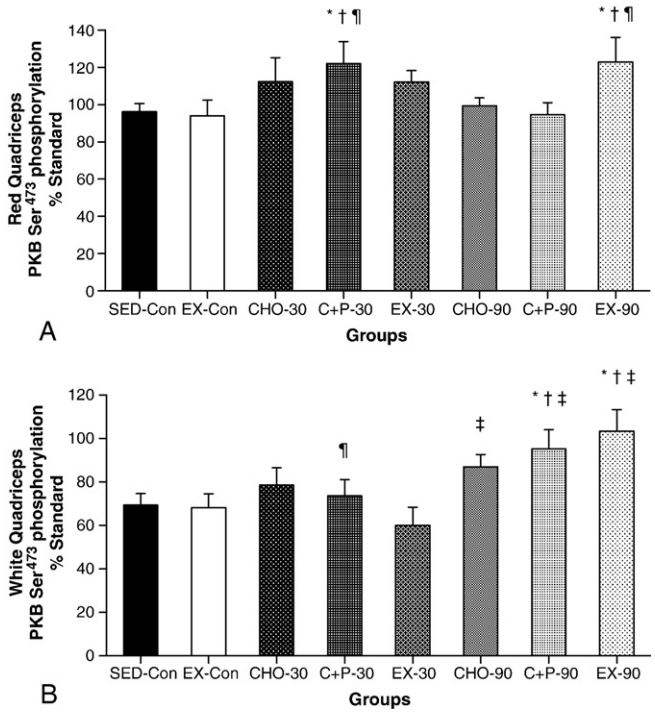

Fig. 3 – Phosphorylation status of PKB Ser473 in red (A) and white (B) quadriceps for SED-Con and exercised rats expressed as percentage of an insulin-stimulated rat tissue standard. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, †from EX-Con, ‡from EX-30, and ¶from C+P-90.

90-minute postexercise groups. However, these groups demonstrated significantly greater PKB phosphorylation compared with the EX-30 group in white quadriceps. Moreover, the C+P-90 and EX-90 groups had significantly greater PKB phosphorylation compared with the SED-Con ($P \leq .05$) and EX-Con ($P \leq .05$) groups in white quadriceps.

3.5. *Glycogen synthase kinase–3*

Immediately following exercise, the phosphorylation state of either GSK-3α Ser21 or GSK-3β Ser9 in either red quadriceps (Figs. 4A and 5A, respectively) or white quadriceps (Figs. 4B and 5B, respectively) was not different compared with the SED-Con group. However, without nutritional supplementation, prolonged exercise resulted in a significant reduction of the GSK-3α phosphorylation at 30 minutes postexercise compared with the SED-Con ($P \leq .05$) and EX-Con ($P \leq .05$) groups in red quadriceps. The reduction in GSK-3α phosphorylation in the red quadriceps was not observed in the EX-90 group. The EX-90 group had significantly greater GSK-3α phosphorylation compared with the EX-30 group (Fig. 4A). This trend was also demonstrated in the GSK-3β phosphorylation, although significance was only found between the SED-Con and EX-30 groups in red quadriceps (Fig. 5A).

Similar to the responses in the red quadriceps, the exhaustive swimming exercise resulted in a significant reduction of the GSK-3α phosphorylation in the EX-30 group compared with the EX-Con group in white quadriceps. This low phosphorylation state was reversed in the EX-90 group, as this group had significantly greater GSK-3α phosphorylation compared with the EX-30 group in white quadriceps (Fig. 4B). A significant reduction of the GSK-3β phosphorylation in the EX-30 group was also observed in white quadriceps (Fig. 5B).

Although there were no differences in the GSK-3α phosphorylation among the 90-minute postexercise groups, phosphorylation state of the EX-30 group was significantly lower than those of the CHO-90 and EX-90 groups in red quadriceps (Fig. 4A). Among the 30-minute postexercise groups, the phosphorylation state of GSK-3β in the EX-30 group was significantly lower than that of the C+P-30 group in red quadriceps. The EX-30 group also had significantly lower GSK-3β phosphorylation compared with the CHO-90 and C+P-90 groups in red quadriceps. The CHO-90 group had 47.2% greater GSK-3β phosphorylation compared with the EX-Con group ($P < .01$) in red quadriceps (Fig. 5A). For white quadriceps, a significantly lower GSK-3α phosphorylation was observed in the EX-30 group compared with the CHO-30 and C+P-30 groups. Furthermore, the phosphorylation state of GSK-3α in

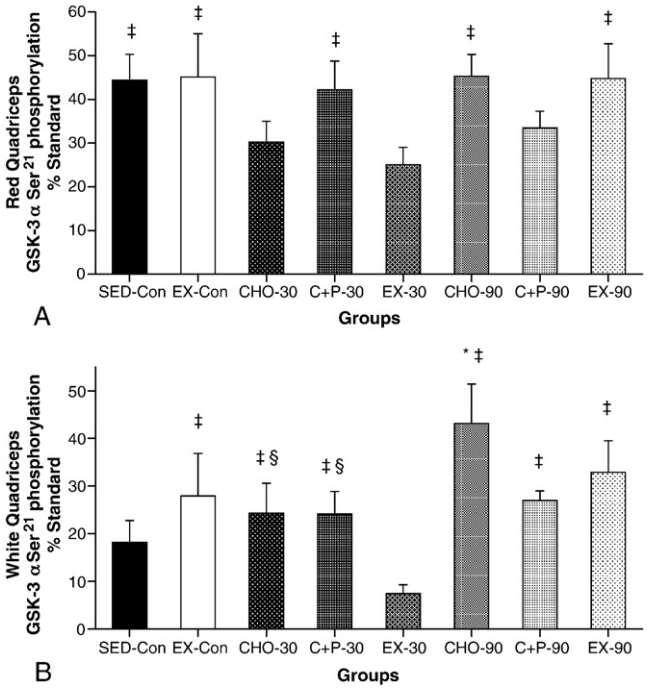

Fig. 4 – Phosphorylation status of GSK-3α Ser21 in red (A) and white (B) quadriceps for SED-Con and exercised rats expressed as percentage of an insulin-stimulated rat tissue standard. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, ‡from EX-30, and §from CHO-90.

the EX-30 group was significantly lower than those of all 90-minute postexercise groups in white quadriceps. In addition, the CHO-90 group had significantly greater GSK-3α phosphorylation compared with the SED-Con, CHO-30, and C+P-30 groups (Fig. 4B). For GSK-3β phosphorylation in the white quadriceps, phosphorylation was significantly lower in the EX-30 group than all the other groups (Fig. 5B).

3.6. *Glycogen synthase*

The prolonged exercise resulted in a 41.0% reduction of GS Ser640 phosphorylation compared with the SED-Con group ($P \leq .01$) in the red but not the white quadriceps (Fig. 6A and B, respectively). Without nutritional supplementation, further reductions of the GS phosphorylation were demonstrated in the EX-30 and EX-90 groups in both red and white quadriceps. These 2 groups had significantly lower GS phosphorylation compared with the SED-Con and EX-Con groups. Although no differences in the GS phosphorylation among the 30-minute postexercise groups were observed, these groups had significantly lower GS phosphorylation compared with the SED-Con, EX-Con, CHO-90, and C+P-90 groups in red quadriceps. Whereas both the CHO-90 and C+P-90 groups had significantly greater GS phosphorylation compared with the EX-90 group, only the C+P-90 group demonstrated significantly greater GS phosphorylation compared with the EX-Con in red quadriceps (Fig. 6A).

In the white quadriceps, there were no differences in the GS phosphorylation among the 30-minute postexercise groups (Fig. 6B). However, these groups had significantly lower GS phosphorylation compared with the SED-Con ($P \leq .01$), EX-Con ($P \leq .01$), and C+P-90 ($P \leq .05$) groups in white quadriceps. No differences in GS phosphorylation were observed among the 90-minute postexercise groups in white quadriceps.

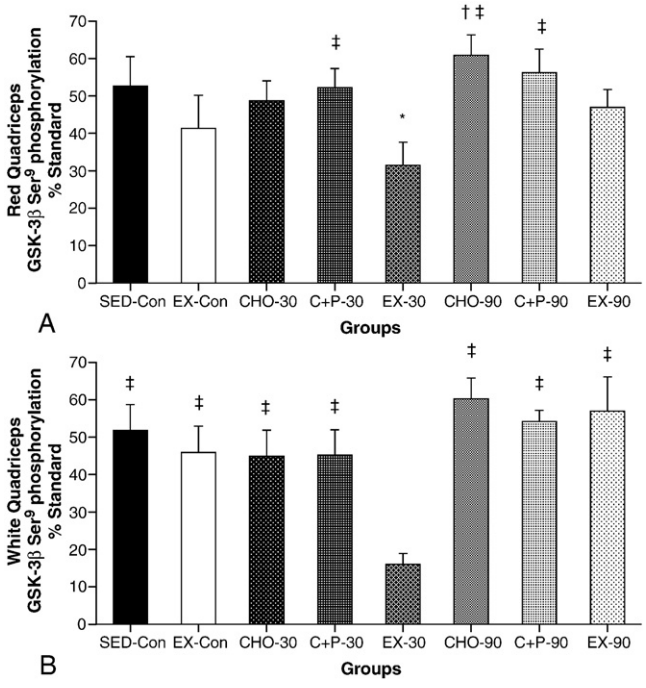

Fig. 5 – Phosphorylation status of GSK-3ß Ser9 in red (A) and white (B) quadriceps for SED-Con and exercised rats expressed as percentage of an insulin-stimulated rat tissue standard. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, †from EX-Con, ‡from EX-30.

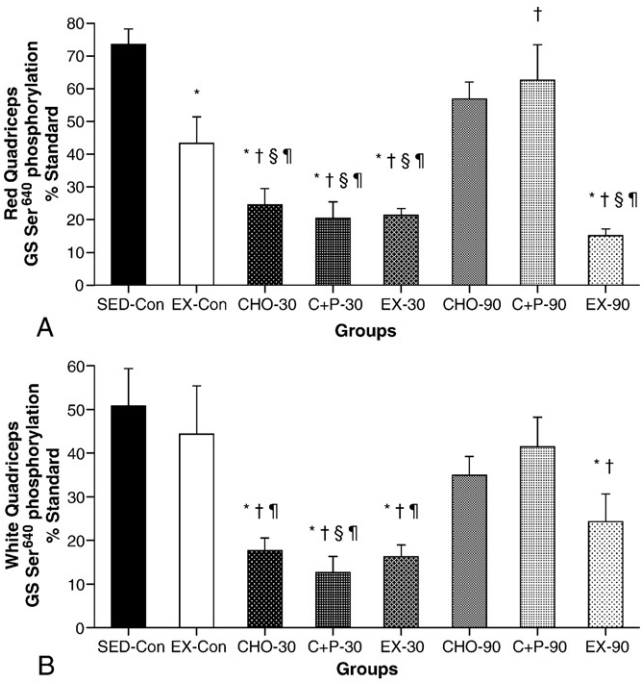

Fig. 6 – Phosphorylation status of GS Ser640 in red (A) and white (B) quadriceps for SED-Con and exercised rats expressed as percentage of an insulin-stimulated rat tissue standard. Exercised rats were either killed immediately after 3-hour swimming exercise (EX-Con) or received carbohydrate (CHO), carbohydrate plus protein (C+P), or no supplement (EX) immediately after exercise and then killed at 30 or 90 minutes postexercise. Values are means ± SE. *Significantly different ($P \leq .05$) from SED-Con, †from EX-Con, §from CHO-90, and ¶from C+P-90.

4. Discussion

The major finding of the present study was that adding protein to a carbohydrate supplement resulted in a significantly greater muscle glycogen concentration compared with carbohydrate alone at 90 minutes postexercise in both the red and white quadriceps of the rat. Furthermore, only the C+P-90 group had stored glycogen to levels of those of the SED-Con group in red quadriceps. These results are in line with similar studies that have shown an increase in glycogen concentration with carbohydrate and protein supplementation [2-5].

Zawadzki et al [4] reported that cyclists provided with a carbohydrate and protein supplement after exercise

demonstrated greater glycogen concentration at 4 hours postexercise compared with those who received carbohydrate alone. Whereas their result showed differences in glycogen concentration between the C+P and CHO, the study did not have equal calorie contents between the 2 treatments. Subsequent studies applying isocaloric supplements have, however, demonstrated that C+P results in greater muscle glycogen synthesis when supplements are provided at 1- to 2-hour intervals [2,3].

The results of the present study, which shows a beneficial effect with the addition of protein to a carbohydrate supplement, can be contrasted to several studies, which have observed no differences in muscle glycogen synthesis between C+P and CHO after recovery from prolonged exercise [9-13]. These inconsistent findings in glycogen synthesis are likely the result of differences in the specific study designs. For instance, some studies have provided subjects with different nutrient doses [12,13] and have used different recovery lengths [9]. Moreover, the type of protein and carbohydrate selected for supplementation as well as the frequency of supplement administration may influence the effectiveness of the supplement [5,13,19,20].

There are 2 phases in glycogen synthesis after prolonged exercise: the insulin-independent phase and the insulin-dependent phase [8,21,22]. During the first phase of glycogen synthesis, insulin is not required; and a rapid stimulus of glycogen synthesis lasts for 30 to 60 minutes. Thereafter, the presence of insulin is necessary for glycogen synthesis [8,21,22]. Surprisingly, in the present study, net glycogen storage was minimal during the first 30 minutes of recovery. Therefore, the greater muscle glycogen storage observed in the C+P-90 group most likely occurred during the insulin-dependent phase.

There are conflicting results in the literature as to the role of the insulin response on glycogen synthesis with differing postexercise supplements. For instance, Zawadzki et al [4] found significant differences in the insulin response after feedings with C+P and with CHO alone. They suggested that greater insulin secretion during the C+P treatment might have been the cause for the differences in glycogen concentration. However, Ivy et al [3] later reported no differences in insulin response among C+P, isocarbohydrate CHO, and isocaloric CHO supplemental treatments when the subjects received the supplements immediately and 2 hours after a cycling exercise. Despite no differences in the insulin response, the subjects still demonstrated greater glycogen synthesis 4 hours postexercise when they were supplemented with C+P. Similar to the results of Ivy et al [3], the present study did not observe any differences in the insulin response between the C+P and CHO treatments. Therefore, in the present study, the faster rate of muscle glycogen storage in the C+P treatment as opposed to the CHO treatment cannot be explain by a greater postsupplement insulin response.

Although the addition of protein to a carbohydrate supplement did not seem to influence the insulin response, it may have induced greater activation of the enzymes regulating glycogen synthesis such as PKB and GS. Therefore, the second purpose of this study was to investigate the differences between supplementing with carbohydrate alone and carbohydrate plus protein on the phosphorylation status of enzymes controlling glycogen synthesis. For each enzyme, the effect of the prolonged exercise was examined first, followed by the comparison of each of the nutritional supplements.

Contradictory results have been reported with respect to the effect of exercise on the phosphorylation status of PKB. The present finding that PKB phosphorylation was not altered by exhaustive exercise is in agreement with several previous studies [23,24]. However, more recent results suggest that PKB is phosphorylated and/or activated by exercise [25-27] and muscle contraction [28-30]. This discrepancy might be caused by differences in the type, intensity, or mode of exercises used in the experimental designs of each study or the timing of tissue sampling. Interestingly, in the present study, the prolonged exercise resulted in a delayed onset of PKB phosphorylation, where a significant increase was observed at 90 minutes postexercise in both red and white quadriceps. These elevations were in line with the return of blood glucose and insulin concentrations to baseline levels, which can clearly be attributed to gluconeogenesis. Because PKB activation is intimately involved with glucose transport, its delayed phosphorylation status could facilitate the return of blood glucose to normal levels by limiting the clearance of blood glucose by skeletal muscle. Such a response would preserve the low circulating levels of glucose for the nervous system until euglycemia was reestablished.

With respect to the effect of supplementation, no differences in PKB phosphorylation were observed between the nutrient groups at any time point examined. However, PKB phosphorylation in the red quadriceps of the C+P rats was increased above SED-Con and EX-Con groups at 30 minutes postexercise and in white quadriceps at 90 minutes postexercise. Phosphorylation of PKB was not increased above SED-Con or EX-Con levels with CHO supplementation alone. The reason for the delayed response in PKB phosphorylation in the white quadriceps following C+P supplementation is not known. The literature does indicate that the phosphorylation status of PKB is variable among fiber types [29]. However, the results suggest that C+P supplementation may have a greater influence on the phosphorylation of PKB postexercise than CHO supplementation. This premise is supported by the recent findings of Morifuji et al [5]. These researchers reported that 2-hour postexercise glucose and glucose plus whey protein hydrolysates increased PKB phosphorylation, but that the increase was greatest after the glucose plus whey protein hydrolysate supplement.

When activated by dephosphorylation, GSK-3 is thought to inhibit glycogen synthesis as well as translation initiation. Although the inactivation of GSK-3α after prolonged exercise has been observed [23,26], activation of this enzyme has also been reported [24]. It has been suggested that inactivation of GSK-3α is dependent on its phosphorylation at Ser21 [26], although this has not always been demonstrated [23]. In contrast to the α-isoform, exercise has not been found to have an effect on GSK-3β activity [24,26]. In the present study, the prolonged swimming exercise had no effect on either isoform of GSK-3 phosphorylation compared with the sedentary group (SED-Con) when evaluated immediately after exercise. However, without nutritional supplementation, phosphorylation

states of GSK-3 in both isoforms were significantly reduced at 30 minutes postexercise. Although a decrease in GSK-3 phosphorylation would be expected to lead to an increase in GS phosphorylation and its inhibition, GS phosphorylation was significantly reduced. The literature reports that muscle glycogen concentration is the primary regulator of GS [31,32]. Therefore, these results suggest that the muscle glycogen concentration has a greater influence over the phosphorylation status of GS than GSK-3.

The results for GSK-3 also indicate that protein synthesis may be suppressed if a nutritional supplement is not provided after prolonged exercise because GSK-3 is an inhibitor of eukaryotic initiation factor (eIF) 2B [33]. Eukaryotic initiation factor 2B (eIF2B) is a guanine nucleotide exchange factor that catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on eIF2. This allows methionyl–transfer RNA to bind to the 40S ribosomal subunit as a ternary complex with eIF2 and GTP, the initial step in translation initiation. Therefore, when blood glucose and plasma insulin levels are low after exhaustive exercise, GSK-3 may inhibit protein synthesis, but have little effect on GS activity because of reduced muscle glycogen stores. Whereas a dephosphorylation of GSK-3 in both isoforms existed at 30 minutes postexercise, their phosphorylation status had recovered by 90 minutes postexercise. The rephosphorylation of GSK-3 appeared to be related to the return of plasma insulin and blood glucose concentrations to within reference range.

Similar to PKB, there were no differences in GSK-3 phosphorylation between nutritional supplements at any time point examined. However, C+P supplementation increased GSK-3 phosphorylation in red quadriceps 30 minutes after exercise (C+P-30 > EX-30), whereas CHO supplementation had no effect. Thus, C+P supplementation may have a beneficial effect on protein synthesis during the early phase of recovery [34-37]. In contrast to these results in red quadriceps, both nutritional supplements prevented the dephosphorylation of GSK-3 in both isoforms at 30 minutes postexercise in white quadriceps.

Muscle glycogen concentration negatively correlates with GS activity [31,37-39]; and therefore, it is not surprising that researchers have found that the activity ratio of GS is increased immediately after cessation of exercise when muscle glycogen concentrations are low [40-42]. In agreement with previous studies, the observed phosphorylation of GS was significantly decreased in red quadriceps immediately after 3 hours of swimming exercise. However, prolonged exercise had no immediate effect on GS phosphorylation in white quadriceps. Without nutritional supplementation, a further reduction of GS phosphorylation was observed in both red and white quadriceps at 30 and 90 minutes postexercise. Although blood glucose and plasma insulin concentrations return to within reference range by 90 minutes postexercise in nonsupplemented rats, phosphorylation of GS remained significantly reduced. These results suggest that postexercise muscle glycogen concentration has a greater effect on GS activity than does insulin availability [31,32].

Nutritional supplementation appeared to have no effect on GS phosphorylation at 30 minutes postexercise. Because adding protein to a carbohydrate supplement resulted in a significantly greater glycogen concentration at 90 minutes postexercise than carbohydrate alone, a greater dephosphorylation of GS was expected in this group during the early phase of recovery. In fact, GS phosphorylation was not different among the CHO-30, C+P-30, and EX-30 groups, suggesting that factors in addition to the phosphorylation status of GS were responsible for differences in rates of glycogen storage from 30 to 90 minutes postexercise among treatments. However, the phosphorylation of GS at 90 minutes postexercise tended to reflect the changes in muscle glycogen from 30 to 90 minutes of recovery. This suggests that the phosphorylation of GS was proceeding in parallel with the increase in muscle glycogen in the supplemented rats.

Although the phosphorylation states of the enzymes that drive glycogen synthesis were measured, the results cannot explain the greater glycogen concentration found by addition of protein to a carbohydrate supplement. Therefore, there may be other intracellular mechanisms involved. In this regard, it has been demonstrated that, under the appropriate circumstances, glucose transport can be rate limiting for muscle glycogen synthesis [43]. Moreover, certain amino acids, particularly leucine and isoleucine, have been reported to increase the phosphorylation of glucose transport signaling proteins such as PKB and atypical protein kinase C and increase skeletal muscle glucose uptake in an insulin-independent manner [44-48]. Moreover, the phosphorylation of these proteins was directly associated with an increase in muscle glycogen storage [5,48]. In the present study, we found that PKB at 30 minutes after exercise in red and 90 minutes after exercise in white quadriceps muscles was increased significantly above sedentary control values by the C+P supplement, whereas CHO supplementation did not have this effect. These results suggest that carbohydrate/protein supplementation postexercise may increase muscle glycogen storage by facilitating the glucose transport process, which supports the findings of Morifuji et al [5,48].

In summary, supplementation immediately postexercise with a combination of protein and carbohydrate resulted in a significantly greater muscle glycogen concentration at 90 minutes postexercise compared with supplementing with only carbohydrate. This greater rate of synthesis occurred in both fast-twitch red and white muscle fibers. These results provide strong evidence that protein can influence carbohydrate metabolism and increase the rate of muscle glycogen synthesis. A weakness of this study is that there was no clear mechanism of action identified for the stimulation of muscle glycogen synthesis by the C+P supplement. Based on the results of our signaling proteins, however, it appears that C+P supplement did not increase GS activity subsequent to inhibition of GSK-3. Thus, our results have narrowed the possibilities by which protein could enhance muscle glycogen storage. One likely possibility that should be investigated is facilitation of the glucose transport system by the C+P supplement.

From a practical view, the present results support the addition of protein to a carbohydrate supplement for increasing muscle glycogen resynthesis and recovery from exercise and other forms of physical exhaustion. This can be of significant benefit to the athlete and also to individuals whose occupations require that they remain in a physically ready state.

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